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(54) Title: LYTIC ENZYMES USEFUL FOR TREATING FUNGAL INFECTIONS

(57) Abstract

The present invention features a new method for isolating and purifying lytic enzymes useful for treating fungal infections from such microorganisms as *Trichoderms*. The present invention further features methods of treating fungal infections in mammals including humans by administering one or more lytic enzymes isolated and purified from a microorganism such as a *Trichoderm* and compositions comprising the same.

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LYTIC ENZYMES USEFUL FOR TREATING FUNGAL INFECTIONS

FIELD OF THE INVENTION

The present invention is in the field of medicine, carbohydrate chemistry and biochemistry. Specifically, the present invention features a new method for isolating and purifying lytic enzymes from such microorganisms as *Trichoderma spp.* The present invention further features methods of treating, fungal infections in animals and humans by administering lytic enzymes and compositions comprising the same.

BACKGROUND OF THE INVENTION

Carbohydrates play many important roles in the functioning of living organisms. In addition to their metabolic roles, carbohydrates are structural components of the cell walls and membranes of plants, animals and microbes. Fungal cell walls commonly contain polymers of D-glucose called glucans and polymers of nitrogen containing N-acetyl-D-glucosamine called chitin. Bartnicki-Garcia, *Ann Review Microbiol.*. 22:87-108 (1968). Glucans may be chemically linked in various ways, most commonly through anomeric (α or β) C1 carbons to the C2, C3, C4 or C6 carbons of a neighboring glucose moiety. This variation in linkage may be expressed as a "β1-3 glucan" or a "β1-6 glucan." Chitins, however, are only linked in a β1-4 fashion. The glucans and chitins of fungal cell wall are primarily involved as structural components, maintaining rigidity and conferring protection. They may also be degraded and used as nutritional sources after exhaustion of external nutrients.

While the majority of the fungi carry out essential activities in nature, some are pathogenic to plants, animals and humans. Fungi cause diseases in animals and humans through several mechanisms. First, some fungi elicit immune responses that can result in allergic reactions. For example, asthma and other hypersensitivity reactions are caused. by exposure to specific fungi antigens in the environment. A second fungal disease-causing mechanism involves toxins generated by fungi. For example, afatoxin produced *by Aspergillus flavus* is highly toxic and induces tumors in some animals. The third mechanism of fungal related disease is through infection. The growth of a fungus on or in the body can cause symptoms that range in severity from relatively innocuous, superficial diseases to serious, life-threatening diseases. In 1996, there were over a million serious fungal infections in the United States. The problem of serious invasive fungal infections has increased with advances in transplantation technology and the growing numbers of patients with immunosuppressive viral infections. *Aspergillus* infections are common in up to 5% of immunosuppressed patients with mortality rates approaching 90%.

Conventional anti-fungal agents include topical antiseptic chemicals used for non-invasive infections and polyene and azole antibiotics which inhibit fungal cell wall sterol biosynthesis. Polyenes produced by streptomyces species bind to ergosterol (equivalent of cholesterol in higher eukaryotic cell membranes), which disrupts the normal membrane function and eventually causes membrane permeability and cell death. Azole is a group of antibiotics that selectively inhibit ergosterol biosynthesis. The treatment with azoles results in the inability of fungi to produce a normal membrane, leading to membrane damage and alteration of critical membrane activities. Polyenes and azoles have satisfactory effects on common species of fungi such as *Candida, Histoplasma* and *Coccidiodes,* but they exhibit little activity against the invasive *Aspergillus* which are a common cause of death in the immunosuppressed population. In addition, treatment of fungal infection based on polyenes and azoles is often toxic and has many undesirable side effects. The use of the existing anti-fungal agents has also resulted in the emergency of populations of resistant fungi and the emergence of new pathogenic fungi strains.

Thus, it is advantageous to provide novel antibiotics with different mechanisms of action from those in the prior art. These novel antibiotics may be used to broaden the scope of anti-fungal treatment and complement the activities of known anti-fungal agents. They may also be used to treat fungal infections in humans and animals which are resistant to conventional drugs. The use of lytic enzymes to degrade fungal cell walls as the basis for anti-fungal treatment in animals and humans is the focus of the instant invention.

Many fungi secrete lytic enzymes into their environment as a means to generate food sources from polysaccharides or to gain competitive advantage in their microenvironment by inhibiting the growth of other fungi or parasitizing, their neighbors. Haran *et al., Microbiology*, 142:2321-2331 (1996). Archer *et al., Crit. Rev. Biotechnol.* 17(4):273-306 (1997). The inhibition of fungal growth is attributed to lytic enzymes that degrade fungal cell walls and eventually lead to fungal cell lysis. Fungal lytic enzymes include glucanases, chitinases, proteases, lipases, and other hydrolytic enzymes (glucanase and chitinase are glucan degrading and chitin-degrading enzymes respectively). These lytic enzymes can be further divided into subcategories according to their modes of degradation reaction and type of linkage(s) they degrade, such as, endo- or exo- enzymes and (1,3)-β- or (1,4)-β- enzymes. Lytic enzymes function in an endo fashion to cleave the polymeric linkage at random sites along the polysaccharide chain. Those that function in an exo- fashion cleave subsequent polymeric units from the end. Glucanases and chitinases such as endo and exo (1,3)-β-glucanases, endo and exo (1,4)-β-glucanases, endo and exo (1,6)-β-glucanases, endochitinases, exochitinases, chitobiohydrolases, endochitosanases, exochitosanases, 1,4-β-poly-N-acetyl-D-glucosaminidase, and endo and exo 1,4-β-poly-D-glucosaminidase have been detected in a wide range of fungi species.

Considerable research effort has focused on the studies of lytic enzymes produced *by Trichoderms.* These are common fungi found in almost any soil. They are strongly antagonistic to other fungi. The antagonism is in part due to their secretion of lytic enzymes, such as glucanases, chitinases and proteases, to degrade cell walls of other fungi and in turn utilize their nutrients. 1,3-β-glucan is one of the main structural components of the fungal cell wall, and 1,3-β-glucanases are secreted by a number of *Trichoderma* species. Kitamoto *et al., Agric. Biol. Chem.* 51:3385-3385 (1987); Dubourdieu *et al., Carbohydr. Res.* 144:277-287 (1985); Lorito *et al., Phytopathology* 84:398-405 (1994); Del Rey *et al., J Gen. Microbiol.* 110:83-89 (1979).

Many of the 1,3-β-glucanases have been extensively characterized and studied, and many of their encoding genes have been identified and cloned. Their involvement in biological control and plant defense mechanisms against fungi has also been well documented. Haran *et al., Microbiology* 142:2321-2331 (1996). 1,6-β-glucanases have been shown to lyse yeast and fungal cell walls. Relatively little information is reported with respect to their purification, characterization, and anti-fungal activities. Haran *et al., Microbiology* 142:2321-2331 (1996). *Trichoderma harzianum* was shown to produce at least two extracellular 1,6-β-glucanases. De la Cruz *et al.* were the first to purify one of the two 1,6-β glucanases to homogeneity and to study their hydrolytic activity against fungal cell walls. De la Cruz *et al., J Bateriol.* 177:6937-6945 (1995). Several chitinases are secreted by *Trichoderma harzianum.* Many of the chitinases have been identified and purified to homogeneity or near homogeneity. Haran *et al., Microbiology* 142:2321-2331 (1996). Several chitinase encoding genes have also been cloned and overexpressed. De La Cruz *et al., Eur. J. Biochem.* 206:859-867 (1992). Some have demonstrated some *in vivo* efficacy with mixtures of mycolases including chitinases, β-1,3-glucanases and exo-glycosidaes. Chalkley *et al., J. Med. Vet. Mycology* 23:147-164 (1985); Pope *et al., Postgraduate Medical Journal* 55:674-676 (1979). It is believed that others have cloned a human chitinase. However, none have successfully purified individual lytic enzymes from microorganisms and demonstrated *in vivo* efficacy of such purified lytic enzymes individually or in combinations of one or more purified lytic enzymes or with such purified lytic enzymes in combination with one or more conventional anti-fungal agents.

A variety of glucases, chitinases, proteases and other hydrolytic enzymes produced by *Trichoderma* species have been implicated in the biological control of plant fungal pathogens. These lytic enzymes have not been used for treating fungal infections *in vivo.* The treatment based on these lytic enzymes disclosed herein offers a new approach to fighting fungal infections, especially against the more invasive and resistant fungal infections. Because humans and animals are not known to

have glucan or chitin structures like those of lower animals and microbes, glucanases, chitinases and proteases should not display significant toxicity or undesirable biologic effects in humans or animals.

SUMMARY OF THE INVENTION

In general, the present invention features a new method for isolating and purifying lytic enzymes useful for treating fungal infections from *Trichoderms*. The present invention further features methods of treating fungal infections in mammals including humans by administering one or more lytic enzymes and compositions comprising the same.

In one embodiment, novel methods for isolating and purifying lytic enzymes from such microorganisms as *Trichoderms* is disclosed. Specifically, the novel methods comprise the steps of (i) precipitating the cellular material from a microorganism such as a *Trichoderma* species, (ii) isolating the proteins therefrom, (iii) precipitating the lytic enzyme by adding its substrate(s), and (iv) purifying the enzyme by isoelectric focusing. The method disclosed herein is applicable to a variety of lytic enzymes from microorganisms, preferably fungii, and especially preferably *Trichoderma* species, and may be practiced on a variety of species to isolate a variety of lytic enzymes without undue experimentation. Exemplary lytic enzymes according to the present invention include glucanases, chitinases, chitosanases and proteases. In preferred embodiments, the present method is applied to isolating and purifying β-1-6 glucanase from *Trichoderma harzianum.*

In a second embodiment, the present invention features methods for treating mammals including humans suffering from fungal or mycoparasitic infections by administering a pharmaceutically effective amount of one or more lytic enzymes such as those obtained from a microorganism such as a *Trichoderm.* Lytic enzymes produced by microorganisms such as *Trichoderms* useful for treating fungal and mycoparasitic infections include glucanases, chitinases, chitosanases and proteases. They may be administered *in vivo* to organisms in order to treat, eliminate or prevent infection by organisms possessing a cell wall such as fungi. Exemplary fungal species that may be treated by the compounds of the present invention include, for example, *Aspergillus* infections.

In a third embodiment, the present invention encompasses cellular transformation vectors containing nucleic acid sequences encoding therapeutic lytic enzymes from such microorganisms as *Trichoderma* species. The enzymes according to the present invention may be administered by genetic therapy techniques wherein a nucleotide encoding the therapeutic compound is administered to a cell or to an organism in order to produce the therapeutic compound endogenously. Those of skill in the art will appreciate many methods for administering transformation vectors containing nucleic acid sequences encoding therapeutic lytic enzymes.

In a fourth embodiment, the present invention features pharmaceutical compositions containing lytic enzyme(s) useful for treating fungal infections. The lytic enzymes of the present invention may be administered alone or in pharmaceutically acceptable compositions to treat infections caused by organisms sensitive to their activities, such as fungi possessing a cell wall. In preferred embodiments, lytic enzymes isolated from *Trichoderma harzianum* are particularly effective against fungal infection. In particularly preferred embodiments, a β-1,6-glucanase isolated from *Trichoderma harzianum* has demonstrated anti-fungal activity in mammals against *Aspergillus* infection.

Such compositions may be formulated so as to be adapted to the specific method of administration. Such compositions may be optimized for administration of the enzyme by parenteral, topical or oral administration. Additionally, the enzyme may be administered by cellular transformation vectors containing nucleic acid sequences encoding therapeutic lytic enzymes.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 demonstrates the survival rate after glucanase treatment of *Aspergillus* infection in mice.

Figure 2 illustrates the survival statistics for up to 18 days post inoculation among subjects treated with PBS intraperitoneal, chitinase intraperitoneal at a dosage of 100 mg/kg, chitinase intravenous at a dosage of 100 mg/kg, chitinase intraperitoneal at a dosage of 25 mg/kg and amphotericin B intraperitoneal at a dosage of 1 mg/kg.

DETAILED DESCRIPTION OF THE INVENTION

The present invention features novel methods for isolating and purifying lytic enzymes from microorganisms such as *Trichoderms*. Moreover, the present invention features methods of treating fungal infections in mammals including humans by administering lytic enzymes and compositions comprising the same.

The novel methods for isolating and purifying lytic enzymes from such microorganisms as *Trichoderms* comprise the steps of (i) precipitating the cellular material from a microorganism such as a *Trichoderma species,* (ii) isolating the proteins therefrom, (iii) precipitating the enzyme by addition of its substrate(s), and (iv) purifying the enzyme by isoelectric focusing. The lytic enzymes that may be isolated and purified by the present invention include glucanases, chitinases, chitosanases and proteases. Exemplary lytic enzymes include, but are not limited to endo and exo (1,3)-β-glucanases, endo and exo (1,4)-β-glucanases, endo and exo (1,2)-β-glucanases, endo and exo (1,6)-β-glucanases, endochitinases, exochitinases, chitobiohydrolases, endochitosanases, exochitosanases,

1,4-β-poly-N-acetyl-D-glucosaminidase, and endo and exo 1,4-β-poly-D-glucosaminidase. The invention is also specifically intended to encompass lytic enzymes from *Trichoderms* such as, but not limited to, *T. atroviride, T. cirtinoviride, T. hamatum, T. harzianum, T. koningii, T. lignorum, T. longibrachiatum, T. polysporum, T. pseudokoningii, T. reesei, T. saturnisporum, T. todica, T. virgatum* and *T. viride.* In some preferred embodiments, the present method has been successfully applied to isolating and purifying a β 1-6 glucanase enzyme from *Trichoderma harzianum.* This particular enzyme has a molecular weight of about 43,000 daltons and an isoelectric point of about 5.8.

The present invention also provides novel methods and compositions for treating a variety of fungal and other microbial diseases in mammals including humans by administering one or more lytic enzymes according to the present invention. The present invention differs substantially from many other forms of medical therapy for fungal infections because conventional therapy methods use small molecules that inhibit fungal cell wall and membrane sterol synthesis. Because of the mechanisms of action, the existing methods have significant toxicity and side effects in the recipients. The instant invention features a new mechanism for treating fungal infections by administering a pharmaceutically effective amount of one or more lytic enzymes useful to degrade fungal cell walls and to eventually cause fungal cell lysis and death. Such lytic enzymes may be isolated and purified from microorganisms, preferably from fungii and especially preferably from *Trichoderma* species. The instant method is especially designed to target invasive forms of fungal infection for which the existing methods are not optimally effective. In addition, the instant method is also effective in treating fungal infections that are resistant to the existing methods of treatment. In preferred embodiments, a β 1-6 glucanase enzyme isolated from *Trichoderma harzianum,* shown to possess potent anti-fungal activity *in vitro* assays, is administered. In some preferred embodiments, a lytic enzyme according to the invention is administered to effectively treat *Aspergillus* infection.

In another aspect, the present invention also provides pharmaceutical compositions comprising one or more lytic enzyme(s). Therapeutic enzymes may be administered in a number of ways such as parenteral, topical, intranasal, inhalation or oral administration. In some embodiments, the invention provides for administering the enzyme in a pharmaceutical composition together with a pharmaceutically-acceptable carrier which may be solid, semi-solid or liquid or an ingestible capsule. Examples of pharmaceutical compositions useful in the present invention include tablets and drops, such as nasal drops. Compositions for topical application include, but are not limited to ointments, jellies, creams and suspensions, aerosols for inhalation, nasal spray, and liposomes. One or more lytic enzyme will comprise between 0.05 and 99% or between 0.5 and 99% by weight of the

composition. In preferred embodiments, the enzyme content may be between 0.5 and 20% for injection and between 0.1 and 50% for oral administration.

To produce pharmaceutical compositions for oral application containing the therapeutic lytic enzyme(s), the enzyme(s) may be mixed with a solid, pulverulent carrier. The carrier may include, but is not limited to lactose, saccharose, sorbitol, mannitol, a starch (for example, a potato starch or a corn starch), amylopectin, laminaria powder, citrus pulp powder, a cellulose derivative and gelatine. The pharmaceutical compositions may also include lubricants such as magnesium or calcium stearate or a Carbowax or other polyethylene glycol waxes, and they may be compressed to form tablets or cores for dragees. If drakes are required, the cores may be coated with, for example, a concentrated sugar solution. The sugar solutions may contain gum arabic, talc and/or titanium dioxide, or alternatively a film forming agent dissolved in easily volatile organic solvents or mixtures of organic solvents. Dyestuffs may be added to such coatings, for example, to distinguish between different contents of active substance. For a composition of soft gelatine capsules consisting of gelatine, or glycerol as a plasticizer, or similar closed capsules, the active substance may be admixed with a Carbowax® or a suitable oil such as sesame oil, olive oil, or arachis oil. Hard gelatine capsules may contain granulates of the active substance with solid, pulverulent carriers such as lactose, saccharose, sorbitol, mannitol, starches (for example, potato starch, corn starch or amylopectin), cellulose derivatives or gelatine, and they may also include magnesium stearate or stearic acid as lubricants.

Therapeutic lytic enzymes of the present invention may also be administered parenterally such as by subcutaneous, intramuscular or intravenous injection or by sustained release subcutaneous implant. In subcutaneous, intramuscular and intravenous injection, a therapeutic enzyme or other active ingredient may be dissolved or dispersed in a liquid carrier vehicle. For parenteral administration, the active material may be suitably admixed with an acceptable vehicle, preferably of the vegetable oil variety such as peanut oil, cottonseed oil and the like. Other parenteral vehicles such as organic compositions using solketal, glycerol, formal, and aqueous partnered, formulations may also be used. For parenteral application by injection, compositions may comprise an aqueous solution of a water soluble pharmaceutically acceptable salt of the active acids according to the invention, desirably in a concentration of 0.5-10%, and optionally also a stabilizing agent and/or buffer substances in aqueous solution. Dosage units of the solution may advantageously be enclosed in ampoules. When therapeutic enzymes are administered in the form of a subcutaneous implant, the compound may be suspended or dissolved in a slowly dispersed material known to those skilled in the art or administered in a device which slowly releases the active material through the use of a constant driving force such as an osmotic pump. In such cases, administration over an extended period of time may be possible.

For topical application, the pharmaceutical compositions are suitably in the form of an ointment, gel, suspension, cream or the like. The amount of active substance may vary, for example between 0.05-20% by weight of the active substance. Such pharmaceutical compositions for topical application may be prepared in known manners by mixing the active substance with known carrier materials including but not limited to isopropanol, glycerol, paraffin, stearyl alcohol, polyethylene glycol. The pharmaceutically acceptable carrier may also include a known chemical absorption promoter. Examples of absorption promoters are dimethylacetamide (U.S. Patent No. 3,472,931), trichloro ethanol or trifluoroethanol (U.S. Patent No. 3,891,757), certain alcohols and mixtures thereof (British Patent No. 1,001,949). A carrier material for topical application to unbroken skin is also described in the British patent specification No. 1,464,975, which discloses a carrier material consisting of a solvent comprising 40 70% (v/v) isopropanol and 0 60% (v/v) glycerol, the balance, if any, being an inert constituent of a diluent not exceeding 40% of the total volume of solvent.

The dosage at which pharmaceutical compositions containing one or more lytic enzymes are administered may vary within a wide range and depends on various factors, such as the severity of the infection and the age of the patient. The dosage may have to be individually adjusted. In preferred embodiments, the amount of therapeutic enzyme is from about 0.1 mg to about 2,000 mg or from about 0.1 mg to about 2,000 mg per day. The pharmaceutical compositions containing a therapeutic lytic enzyme may suitably be formulated so that they provide doses within these ranges either as single dosage units or as multiple dosage units. In addition to containing a therapeutic lytic enzyme (or therapeutic lytic enzymes), the pharmaceutical compositions may contain one or more substrates or cofactors for the reaction catalyzed by the therapeutic enzyme in the compositions.

The therapeutic lytic enzymes according to the present invention may be administered by means of transforming patient cells with nucleic acids encoding a therapeutic enzyme when the therapeutic enzyme is a protein or ribonucleic acid sequence. A nucleic acid sequence encoding a therapeutic lytic enzyme may be incorporated into a vector for transformation into cells of a subject to be treated. A vector may be designed to integrate into the chromosomes of the subject, for example, retroviral vectors, or to replicate autonomously in the host cells. Vectors containing nucleotide sequences encoding a therapeutic lytic enzyme may be designed to provide for continuous or regulated expression of the enzyme. Additionally, the genetic vector encoding the therapeutic enzymes may be designed to stably integrate into the cell genome or to only be present transiently. The general methodology of conventional genetic therapy may be applied to polynucleotide sequences encoding therapeutic enzymes. Reviews of conventional genetic therapy techniques can be found in Friedman, *Science* 244:1275-1281 (1989); Ledley, *J Inherit. Metab. Dis.* 13:587-616 (1990); and Tososhev *et al., Curr Opinions Biotech.* 1:55-61 (1990).

EXAMPLES OF THE PREFERRED EMBODIMENTS

The following examples further illustrate the present invention. These examples are intended merely to be illustrative and are not to be construed as limiting.

EXAMPLE 1

Isolation of βl-6 Glucanase from *Trichoderma harzianum*

Procedure:

Culture of *Trichoderma harzianum* for enzyme isolation was accomplished under the following growth conditions. *T. harzianum* (ATCC 52324) was obtained from the American Type Culture Collection (Rockville, MD). The lyophilized pellet was re-suspended in modified Czapek medium (250 ml containing 0.2 mg/L $MgSO_4 \bullet 7H_20$, 0.9 mg/L KH_2PO_4, 0.2 mg/L KC1, 1.0 mg/L NH_4NO_3, and 0.002 mg/L Zn^{++}) supplemented with 10% glucose. The culture was allowed to grow for 48 hours at 24°C with aeration. The culture was filtered, and the cells were re-suspended in the media described above (1.0 L) except that 1.5% chitin was substituted for the 10% glucose. The culture was incubated for four days at 24°C with aeration.

Isolation of lytic enzymes was performed at 4°C. Following the incubation, the cells were filtered through a filter paper (Whatman No. 1) and the filtrate was centrifuged at 6,000 x g for 10 minutes. The supernatant was precipitated with ammonium sulfate to 80% saturation. The precipitate was recovered by centrifugation at 12,000 x g for 20 minutes and re-suspended in distilled water. The mixture was then dialyzed against 50 mM potassium acetate buffer, pH 5.5. The dialyzed fraction contained lytic enzymes.

The crude enzyme (10 g) was dissolved in water (100 ml) and dialyzed against sodium acetate buffer, pH 5.0. The dialyzed enzyme was adsorbed on alcohol precipitated pustulan (βl-6 glucan, 5.0 g) at 4°C for 20 minutes. The supernatant containing non-adsorbed enzyme was collected by centrifugation and readsorbed on fresh pustulan. The process was repeated for three times. All pustulan-enzyme precipitates were pooled and washed three times with sodium acetate buffer (pH 5.0, 100 ml) containing 1M sodium chloride. The pustulan-enzyme complex was then re-suspended in phenylmethylsulfonyl fluoride (lmM) with 0.02% sodium azide and incubated overnight at 37°C to digest and release the enzyme. All clarified supernatants obtained after pustulan-enzyme incubation treatment were pooled and centrifuged at 12,000 x g for 10 minutes. The supernatant was dialyzed against sodium acetate buffer, pH 5.0. This final preparation was then subjected to preparative isoelectric focusing using ampholytes from pH 5-7 in a Rotofor® unit. Isoelectric focusing was run

at 12 watts at 4°C for 3 hours. Fractions were collected, pooled, concentrated and dialyzed. Purified enzyme was used for physicochemical characterization and anti-fungal testing.

Results:

The final preparation product, β1-6 Glucanase, appeared as a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with an apparent molecular weight of 43,000 daltons and an isoelectric point of 5.8. The purified enzyme had a pH optima of 5.0 and temperature optima of 40-50°C. The enzyme was highly specific in cleaving only β1-6 linked polymers of glucose as demonstrated in Example 2 below producing β1-6 polymers of glucose of greater than 2 units from pustulan.

EXAMPLE 2

Characterization of Specific βl-6 Glucanase Enzyme Activity

Procedure:

The activity of β1-6 glucanase was determined by incubating the enzyme (0.3 ml) in 1% pustulan in 50 mM sodium acetate buffer (pH 5.0, 0.1 ml) for 10 minutes at 37°C. The reaction was stopped by adding dinitrosalicylic acid (0.75 ml). The reducing power of the digest was measured. One unit of enzyme is the amount of enzyme that forms an increase of reductive power equivalent to 1 μm of glucose per minute.

Results:

The crude enzyme was found to contain 2 units per milligram of protein. The final purified enzyme was found to contain 20 units per milligram of protein, or approximately 100-fold purification.

EXAMPLE 3

Characterization of Anti-Fungal Activity

Procedure:

The anti-fungal activity of the β1-6 glucanase was determined in 96-well microtiter plates using RPMI-1640 medium with glutamine (150 ml) and a spore suspension of approximately 1,000 spores of *Aspergillus fumigatus* in the same R.PMI-1640-glutamine medium (50 ml). β1-6 glucanase enzyme (0.008-0.08 units) in 50 mM sodium acetate buffer (pH 5.0) was added. Control buffer without enzyme was also used. The microtiter plate was incubated at room temperature for 18 hours and then transferred to a 37°C incubator. Growth of fungal hyphae was monitored using an inverted microscope.

Results:

The control wells in the plate had good growth of hyphae with over 50 hyphal colonies per well. The crude enzyme preparation was similar to the control, but the purified enzyme showed only minimal hyphal growth with fewer than 5 hyphal colonies per well.

EXAMPLE 4

Characterization of Anti-Fungal Activity in Microorganisms and Animals

Procedure:

Fungal Organisms. A. fumigatus phialoconidia (conidia)were used as infectious particles throughout this study. Isolates of *A. fumigatus* originally obtained from patients were maintained on potato dextrose agar for spore and conidia harvesting. Spores or conidia were harvested in saline and vortex-mixed to break up clumps. The mixture was filtered through eight layers of cheesecloth and washed three times in saline. The concentrate was examined by light microscopy. Spore suspensions were free of hyphal fragments. Viability counts for the production of inocula were determined on Sabouraud's a-agar. The viability of spores or conidia was always >95%.

Induction of Immunodeficiency and Cortisone Acetate Treatment. Four- to six-week-old female pathogen-free mice (CD-1 strain) were obtained from Charles River Breeding Laboratories (Kingston, NY). The mice were given free access to water and a standard laboratory diet until 8 hr before cyclophosphamide or buffer injection, when food was withdrawn. Cyclophosphamide was used to induce immune suppression. Briefly, cyclophosphamide was dissolved in ice-cold citrate buffer (pH 4.2). A dose of 250 mg/kg, (0.2 ml) was injected intraperitoneally within 10 min of dissolution. Control animals received buffer (0.2 ml). Mice were used in the experiment 7-14 days after cyclophosphamide or buffer injection. Cortisone acetate was injected subcutaneously in a daily dose of 125 mg/kg in 0.15 M NaCl solution (0.1 ml) for six consecutive days just before challenge. Control animals received NaCl solution alone (0.1 ml).

Animal Models. Graded doses (100 to 10 million) of spores, conidia, or sterile aqueous inocula were administered intravenously 7 days after the injection of cyclophosphamide or buffer or on the day after completion of the cortisone acetate or buffer treatment. Animals were observed for 15 days and the LD 50 determined. When the animals died or were killed, the organ distribution of viable fungi was determined. Portions of lung tissue were processed and stained with Grocott methenamine silver and hematoxylin and eosin for histological evaluation (Waldorf *et al., J. Infectious Disease* 150:752-760 (1984)).

Enzyme Treatment. Thirty minutes after intravenous inoculation with spores or conidia, animals were administered intravenously either normal saline (0.1 ml) as controls or a β1-6 glucanase solution (0.15 ml, 2 units of activity). Treatment continued every 24 hours for 5 days.

Results:

There were 8 control animals and 5 enzyme-treated animals. The survival curve is illustrated in Figure 1. The test of significance for the result was a p value of <0.05 (2-tailed Wilcoxon Rank-Sum analysis). The results showed 60% of mice treated with β1-6 glucanase were surviving 15 to 20 days post inoculation. None of the untreated mice were surviving at the same time.

EXAMPLE 5

Isolation of Chitinase from *Trichoderma harzianum*

Procedure:

Fungal organisms. Trichoderma harzianum phialoconidia (conidia) were used as infectious particles. Isolates of *A. fumigatus* originally obtained from patients were maintained on potato dextrose agar for spore and conidia harvesting. Spores of conidia were harvested in saline, vortex mixed to break up clumps, filtered through eight layers of cheesecloth, washed three times in saline, and examined by light microscopy. Spore suspensions were free of hyphal fragments. Viability counts for the production of inocula were determined on Sabouraud's agar. The viability of spores or conidia was always >95%.

Enzyme production and purification

Enzymes were produced using strain PI of *Trichoderma harzianum* (ATCC 74058). The strain was grown for 4 days on a rotary shaker in Richard's modified medium, which contained 10 g of KNO_3, 5 g of KH_2PO_4, 2.5 g of $MgSO_4$-$7H_2O_2$ mg of $FcCI_3$, 1% (w/v) crab shell chitin (Sigma), 1% polyvinylpyrrolidone (Polyclar AT, GAF Corp., Wayne, NJ), 150 ml of V8 juice, and 1,000 ml of H_2O at pH 6.0. The biomass was removed by filtration, the supernatant dialyzed against 50 mM potassium phosphate buffer (pH 6.7), and enzymes separated by gel filtration chromatography in a chromatography column packed with Sephacryl S-300 (Pharmacia LKB Biotechnology, Upsala, Sweden), followed by chromatofocusing. A single protein with endochitinase activity was obtained. Purity was confirmed by using native and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) (PhastSystem, Pharmacia) and by isoelectric focusing (IEF). The fractions containing chitobiosidase activity were further separated in a Rotofor IEF cell (Bio-Rad, Richmond, CA). Peak fractions containing only chitobiosidase activity were collected, dialyzed against distilled water, and concentrated to dryness in a SpeedVac apparatus (Savant Instruments, Farmingdale, NY). PAGE, followed by staining, with Coomassie blue, indicated a single protein band. Protein

concentration in the enzyme preparations was determined using the Micro BCA protein assay (Pierce, Rockford, IL) with trypsin inhibitor from soybean (Sigma) as the standard protein. Enzyme solutions were kept at 4°C and utilized for the bioassays within 2 weeks or dried in a SpeedVac apparatus and stored at -20°C until used.

Induction of immunodeficiency and cortisone acetate treatment. Four- to six-week-old female pathogen-free mice (CD-1 strain) were obtained from Charles River Breeding Laboratories (Kingston, NY). The mice were given free access to water and standard laboratory diet until 8 hours before cyclophosphamide or buffer injection, when food was withdrawn. Cyclophosphamide was used to induce immune suppression. Cyclophosphamide was dissolved in ice-cold citrate buffer (pH 4.2), and a dose of 250 mg/kg in 0.2 ml was injected intraperitoneally within 10 minutes of dissolution. Control animals received 0.2 ml of buffer. Mice were used in the experiment 7-14 days after cyclophosphamide or buffer injection. Cortisone acetate was injected subcutaneously in a daily dose of 125 mg/kg in 0.1 ml of 0.15 M NaCl solution for 6 consecutive days just before challenge. Control animals received 0.1 ml of NaCl solution alone.

Animal Model. Graded doses (100 to 10 million) of spores, conidia, or sterile aqueous inocula were administered intravenously 7 days after the injection of cyclophosphamide or buffer or on the day after completion of the cortisone acetate or buffer treatment. Animals were observed for 15 days, and the LD 50 was determined. When the animals died or were killed, the organ distribution of viable fungi was determined. Portions of lung tissue were processed and stained with Grocott methenamine silver and hematoxylin and eosin for histological evaluation (Waldorf *et al., J. Infectious Disease* 150:752-760 (1984)).

Enzyme Treatment. Thirty minutes after intravenous inoculation with spores or conidia, animals were administered intravenously either 0.1 ml of normal saline (controls) or 0.15 ml containing 2 units of chitinase. Treatment continued every 24 hours for 5 days. In all experiments, treatment starts 30 min after infection and daily thereafter until mice are dead or moribund (or until untreated controls are all dead or moribund).

Results:

The survival statistics for 18 days post inoculation among subjects treated with PBS intraperitoneal, chitinase intraperitoneal at a dosage of 100 mg/kg, chitinase intravenous at a dosage of 100 mg/kg, chitinase intraperitoneal at a dosage of 25 mg/kg, and amphotericin B intraperitoneal at a dosage of 1 mg/kg are provided below in Table 1. These data are presented graphically in Figure 2.

Sheet 1

Days	PBS ip	Chitinase ip 100 mg/kg	Chitinase iv 100 mg/kg	Chitinase ip 25 mg/kg	Amp B ip 1 mg/kg
1	100	100	100	100	100
2	100	100	100	100	100
3	100	100	100	100	100
4	100	100	100	100	100
5	100	100	100	100	100
6	90	90	80	80	100
7	80	70	60	80	100
8	70	70	60	40	80
9	60	70	60	40	80
10	40	70	60	40	80
11	0	70	40	20	70
12	0	60	30	20	70
13	0	60	20	0	70
14	0	60	20	0	70
15	0	60	20	0	70
16	0	20	0	0	60
17	0	20	0	0	60
18	0	20	0	0	60

EXAMPLE 6

Chondroitinase B Standard Operating Procedure

Source

The chondroitinase B sequence was retrieved from *Flavobacterium heparinum* (ATCC 13125) DNA by PCR amplification. Primers were designed based on a sequence previously deposited in Genbank- (Accession U27584). The signal peptide was not included within the amplified coding sequence. Primer 5'-terminal restriction sites allowed direct cloning into the thioredoxin-fusion plasmid pThioHis (Invitrogen) cut with NcoI and EcoRI. Expression was optimized in *E. Coli* strain BL21.

Materials Needed

Cell Culture

LB/carbenicillin plate with pThioHisChondB in BL21 cells 50 ml sterile
LB/carbenicillin (60 μg/mL) in a 250 ml flask 4 x 1.0 L sterile
LB/carbenicillin in 2.8 L flasks with >4 cm stir bars
20 mL 200 mM IPTG
4 stir-plates, 4 large trays (for ice), 4 styrofoam platforms 2 x 500 mL centrifuge bottles

Lysis	50 mM Tis-HC1, pH 8.0, 5 mM imidazole, 1 mM EDTA, 1 mM Pefabloc SC, 2 μg/mL Leupeptin Dry ice-ethanol bath 2 x 35 mL Oak Ridge centrifuge tubes
Column	Heparin-acrylic bead column (10-20 mL/L culture media) 500 mL 50 mM Tris-HC1, pH 8.0 40 mL 50 mM Tris-HC1, pH 8.0,150 mM NaC1

Standard Operating Procedure

Preparation

Autoclave 2.8 L flasks with LB media and stir bars. Add carbenicillin after cooling. Media can be stored at room temperature for a day or two. Pour a heparin bead column and wash with 2 column volumes of >1 M NaC1 and at least 3 column volumes of 50 mM Tris-HC1, pH 8.0. Inoculate a single-colony of pThioHisChondB in BL21 cells into 100 mL of LB/carbenicillin. Incubate at 37°C overnight with 250 rpm shaking. Subculture if stationary.

Cell Culture

Seed 20 mL of overnight culture into each 2.8 L flask. Shake at 37°C with 250 rpm shaking until $0.5<OD_{550}<0.7$. Immediately transfer bottles to ice-filled trays on stir-plates and stir at moderate speed until culture temperature is <10°C (should take about an hour). Add a few drops of Antifoam 289 at this stage to prevent frothing of cultures. Add 5 mL 200 mM IPTG to each culture. Allow 5 minutes for inhibitor to soak in, then remove ice-trays. Allow cultures to stir at room temperature for three hours. Place styrofoam between bottles and stir-plates as the latter will warm up and heat the cultures. Pellet cultures in 2 x 500 mL bottles (8,000 rpm for 10 minutes). Weigh the two pellets (combined weight usually between 5-7 grams).

Cell Lysis

Re-suspend the pellets in a total of 80 mL lysis buffer (protease inhibitors added fresh) and transfer to 2 x 50 mL polypropylene Falcon tubes. Vortex to insure that cells are completely re-suspended and that no clumps remain. Except for column chromatography and where otherwise stated, all subsequent steps should be performed at 4°C. Immediately freeze the cells by immersing in a dry ice-ethanol bath. Allow at least 10 minutes for complete freezing to occur. Quickly thaw the cell solution by immersing the tubes in a 37°C bath and shaking. Place thawed cells on ice and sonicate with Branson 450 microtip using an output of 3-4 and a duty cycle of 100% for at least 3 x

15 seconds. Transfer the lysate to 2 x 35 mL Oak Ridge tubes and clarify by spinning at >10,000 rpm for at least 30 minutes (11,000 rpm seems to be sufficient). Transfer the supernatant back to a single 50 mL Falcon tube and supplement with $MgCl_2$ to 10 mM, RNase A to 10 μg/mL, and DNase I to 10 μg/mL. Rock the tube at 4°C for 30 minutes. The weight of the pellet after lysis is usually about 1/3 that of the original cell pellet. Assay 5 μL and 1 μL supernatant for activity against dermatan sulfate.

Column Chromatography

Load heparin column with entire nucleic acid-depleted supernatant. Allow the sample to flow through the column at a rate of <2.0 mL/minute. Assay 5 μl of the flow-through to ensure capture of the chondroitinase. Wash the column with 50-100 mL 50 mM Tris-HC1, pH 8.0. Check the wash for activity to confirm that the majority of the chondroitinase remains bound. Elute the chondroitinase with 40 mL (or less) of 50 mM Tris-HC1, pH 8.0, 150 mM NaC1. Determine the activity of the eluate. Supplement with a stabilizing agent. Successful additives to the column media that have so far been determined are BSA or ovalbumin at 0.05%. Run a 10% protein gel of the pre-column supernatant, flow-through, wash, and eluate fractions for SDS-PAGE. The molecular weight of thiochondroitinase B is about 66 kD. The column can be regenerated by washing with >1M NaC1 and re-equilibrating, with the 50 mM Tris-HC1, pH 8.0. The matrix can be stored in Tris buffer containing 0.02% thimerosal.

Although the invention has been described with reference to the presently preferred embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

WE CLAIM:

1. A method for purifying a lytic enzyme from a fungus comprising, the steps of-.
   (a) precipitating cellular material of the fungus;
   (b) isolating protein components from the cellular material;
   (c) precipitating the lytic enzyme using its substrate; and
   (d) purifying the lytic enzyme using isoelectric focusing.

2. The method according to claim 1 wherein the lytic enzyme is a β1-6 glucanase.

3. The method according to claim 1 wherein the fungus is a *Trichoderm.*

4. The method according to claim 1 wherein the furious is *Trichoderma harzianum.*

5. The method according to claim 1 wherein the lytic enzyme is selected from the group consisting of Endo-(1-3)-β-N-glucanase, Exo-(l-3)-β-N-glucanase, Endo-(1 -6)-β-N-glucanase, Exo-(1-6)-β-N--glucanase, Endo-(1-4)-p-β-glucanase, Endo-(1-4)-β-N-glucanase, Endo-(1-2)-β-N-glucanase, Endo-(1-2)-β-N-glucanase, 1-4-β-poly-N-acetyl-D-glucosaminidase, a chitinase, a chitobiosidase, a chitobiohydrolase, endo- 1 -4-β-poly-D-glucosaminidase, exo-1-4-β-poly-D-glucosaminidase, and a protease.

6. The method according to claim 1 wherein the lytic enzyme is β1-6 glucanase.

7. The method according to claim 1 wherein the lytic enzyme is a chitinase.

8. The method according to claim 1 wherein the fungus is selected from the group of *Trichoderm* species consisting of *T. atroviride, T. cirtinoviride, T. hamatum, T. harzianum, T. koningii, T. lignorum, T. longibrachiatum, T polysporum, T. pseudokoningii, T reesei, T saturnisporum, T. todica, T virgatum,* and *T. viride.*

9. A pharmaceutical composition comprising one or more lytic enzymes having the ability to lyse fungal cell walls and a pharmaceutically acceptable carrier or diluent.

10. The composition according to claim 9 wherein the lytic enzyme is purified from a fungus.

11. A pharmaceutical composition according to claim 9 wherein the lytic enzyme is selected from the group consisting of Endo-(1-3)-β-N-glucanase, Exo-(1-3)-β-N-glucanase, Endo-(1-6)-β-N-

glucanase, Exo-(1-6)-β-N-glucanase, Endo-(1-4)-β-N-glucanase, Endo-(1-4)-β-N-glucanase, Endo-(1-2)-β-N-glucanase, Endo-(1-2)-β-N-glucanase, 1-4-β-poly-N-acetyl-D-glucosaminidase, a chitinase, a chitobiosidase, a chitobiohydrolase, endo-1-4-β-poly-D-glucosaminidase, exo-1-4-β-poly-D-glucosaminidase, and a protease.

12. The composition according to claim 9 wherein the lytic enzyme is β1-6 glucanase.

13. The composition according to claim 9 wherein the lytic enzyme is a chitinase.

14. A method for treating fungal infections comprising the step of administering a pharmaceutically effective dose of a pharmaceutical composition according to claim 9.

15. The method according to claim 14 wherein the pharmaceutical composition comprises a β1-6 glucanase.

16. The method according to claim 14 wherein the pharmaceutical composition comprises a chitinase.

17. The method according to claim 14 wherein the fungal infection is caused by a species of *Aspergillus*.

18. The method according to claim 14 wherein the fungal infection is caused by a species having a cell wall comprising a β-1,6-glucan.

19. The method according to claim 14 wherein the fungal infection is caused by a species having a cell wall comprising a β-1,4-chitin.

20. A recombinant plasmid comprising a nucleic acid sequence encoding a lytic enzyme having the ability to lyse fungal cell walls or a biologically active fragment thereof.

21. A recombinant plasmid according to claim 20 wherein the lytic enzyme is selected from the group consisting of a βl-6 -glucanase and a chitinase.

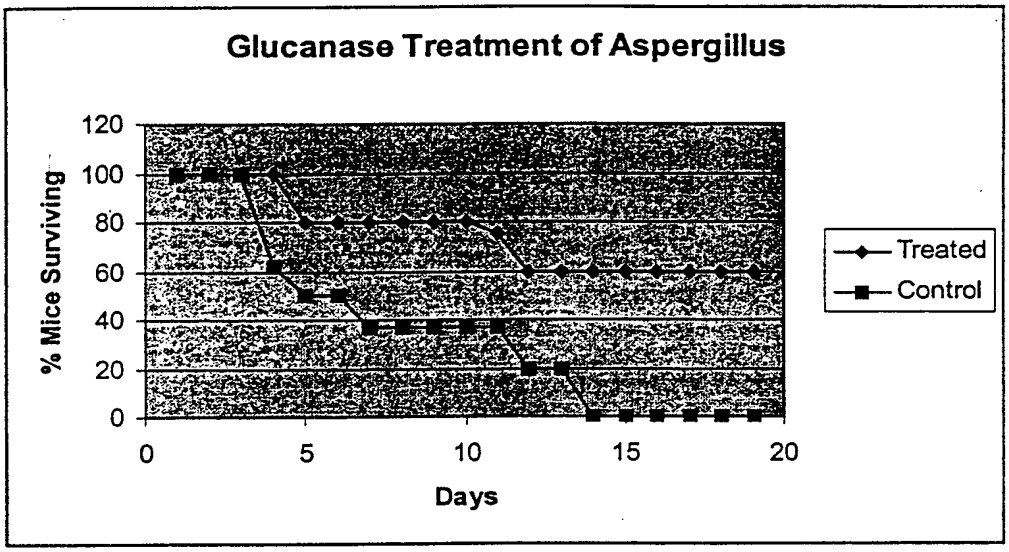
Glucanase Treatment of Aspergillus
% Mice Surviving
120
100
80
60
40
20
0
0
5
10
15
20
Days
Treated
Control

FIGURE 1

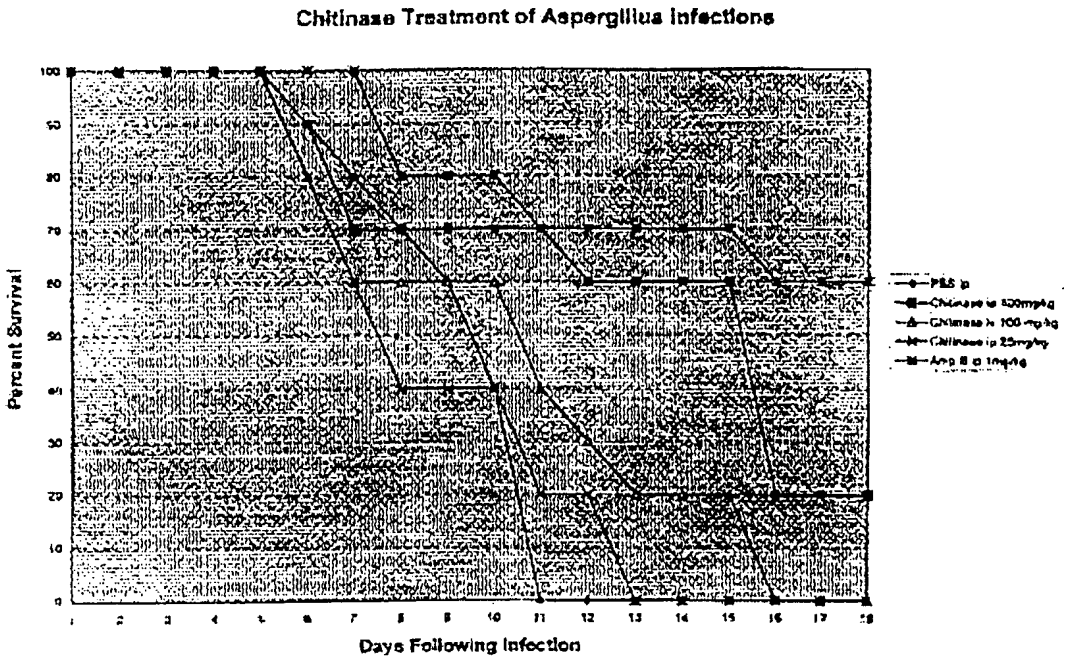
Chitinase Treatment of Aspergillus Infections
Percent Survival
Days Following Infection

FIGURE 2

INTERNATIONAL SEARCH REPORT

Inter· ɔnal Application No
PCT/US 99/10440

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N9/24 C12N9/58 A61K38/47 A61K38/48 C12N15/56
C12N15/57

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CRUZ DE LA J ET AL: "ISOLATION AND CHARACTERIZATION OF THREE CHITINASES FROM TRICHODERMA HARZIANUM" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 206, no. 3, 1 June 1992 (1992-06-01), pages 859-867, XP000615377 ISSN: 0014-2956 cited in the application abstract page 860, right-hand column page 665, right-hand column, paragraph 3 page 866, left-hand column, paragraph 4 - paragraph 5 --- -/--	1,3-8

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 September 1999	24/09/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Ceder, O

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Interr ·nal Application No
PCT/US 99/10440

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 13784 A (CORNELL RES FOUNDATION INC) 23 June 1994 (1994-06-23) page 4, line 6 - line 15 page 5, line 20 - line 21 page 10, line 31 - line 32 page 14 -page 15, line 4 page 16, line 6 - line 18	9-11, 13-19
X	WO 95 31534 A (CHRISTGAU STEPHAN ;KAUPPINEN MARKUS SAKARI (DK); DALBOEGE HENRIK () 23 November 1995 (1995-11-23)	20,21
A	page 2, line 33 -page 3, line 22 page 5, line 27 - line 33 page 6, line 27 - line 31 page 8, line 23 - line 31	9-13
X	WO 94 02598 A (CORNELL RES FOUNDATION INC) 3 February 1994 (1994-02-03) abstract page 2, line 7 - line 24	20,21
A	MULENGA ET AL: "Isolation and characterization of a unique endo-beta-1,6-glucanase from yeast Saccharomycopsis fibuligera NCYC 451" MICROBIOS, vol. 324, no. 80, 1 January 1994 (1994-01-01), page 143 154 XP002076682 ISSN: 0026-2633 abstract	2,12

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/ 10440

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION SHEET PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 14-19 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.1

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

INTERNATIONAL SEARCH REPORT

,ormation on patent family members

Interr ɔnal Application No
PCT/US 99/10440

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9413784 A	23-06-1994	US 5326561 A	05-07-1994
		EP 0684988 A	06-12-1995
		US 5474926 A	12-12-1995
		US 5433947 A	18-07-1995
WO 9531534 A	23-11-1995	AU 2445895 A	05-12-1995
		EP 0759977 A	05-03-1997
		JP 10500296 T	13-01-1998
		US 5770406 A	23-06-1998
WO 9402598 A	03-02-1994	EP 0656059 A	07-06-1995
		JP 7509362 T	19-10-1995
		US 5378821 A	03-01-1995